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의학박사 학위논문

Helicobacter pylori Eradication  
Modulates Aberrant CpG Island  
Hypermethylation in Gastric  
Carcinogenesis

헬리코박터 제균 치료와

위암 관련 유전자의 CpG island 과염기화 억제

2017년 8월

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Helicobacter pylori  
Eradication Modulates Aberrant  
CpG Island Hypermethylation in  
Gastric Carcinogenesis

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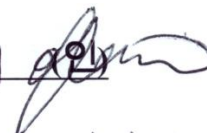
# Helicobacter pylori Eradication Modulates Aberrant CpG Island Hypermethylation in Gastric Carcinogenesis

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# Abstract

**Introduction:** *Helicobacter pylori* (*H. pylori*) infection induces aberrant DNA methylation in gastric mucosa. We evaluated the long-term effect of *H. pylori* eradication on promotor CpG island hypermethylation in gastric carcinogenesis.

**Methods:** *H. pylori*-positive patients with gastric adenoma or early gastric cancer who underwent endoscopic resection were enrolled. According to *H. pylori* eradication after endoscopic resection, the participants were randomly assigned to *H. pylori* eradication or non-eradication group. *H. pylori*-negative gastric mucosa from normal participants provided the normal control. CpG island hypermethylation of tumor-related genes (p16, CDH1, and RUNX-3) was evaluated by quantitative MethyLight assay in non-tumorous gastric mucosa. The gene methylation rate and median values of hypermethylation were compared after one year by *H. pylori* status.

**Results:** In *H. pylori*-positive patients, hypermethylation of p16 was found in 80.6%, of CDH1 in 80.6%, and of RUNX-3 in 48.4%. This is significantly higher than normal control (p16, 10%; CDH1, 44%;

RUNX-3, 16%) ( $p < 0.05$ ). In the *H. pylori* eradication group, methylation rates of p16 and CDH1 decreased in 58.1% and 61.3% of the patients, and the median values of hypermethylation were significantly lower at one year compared with the non-eradication group. However, RUNX-3 hypermethylation did not differ significantly at one year after *H. pylori* eradication. The non-eradication group hypermethylation did not change after one year.

**Conclusions:** *H. pylori* infection was associated with promotor hypermethylation of genes in gastric carcinogenesis, and *H. pylori* eradication might reverse of p16 and CDH1 hypermethylation.

**Keywords:** *Helicobacter pylori*; Eradication; CpG hypermethylation; p16; CDH1; Carcinogenesis

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# I . Introduction

*Helicobacter pylori* (*H. pylori*) infection is one of the most prevalent infectious diseases worldwide and 40–50% of the global human population is estimated to be infected. *H. pylori* has been identified as group I carcinogen by the World Health Organization International Agency for Research on Cancer and is associated with the development of gastric cancer.<sup>1</sup>

Aberrant DNA methylation is one of the most frequent epigenetic changes, which usually takes place at the 5'position of the cytosine ring within CpG dinucleotides, and its influence is the gene silencing and noncoding genomic regions.<sup>2</sup> Promotor CpG island hypermethylation is an crucial mechanism for the silencing of tumor suppressor genes.<sup>3</sup> Aberrant CpG island hypermethylation occurs early in the multi-stage carcinogenesis. Gastric cancer is known to be linked to tumor suppressor-related genes that are inactivated with by CpG island hypermethylation.<sup>4</sup> CpG island hypermethylation has been found in the adjacent noncancerous tissues of gastric cancer patients as well as normal gastric mucosa.<sup>5</sup>

*H. pylori* infection induces aberrant DNA methylation in gastric



mucosa, which causes increase in the gastric cancer risk.<sup>6</sup> Aberrant DNA methylation could be suppressed by *H. pylori* eradication.<sup>7</sup> However, it is still unknown that suppression of aberrant DNA methylation could last over the long-term. We aimed to evaluate long-term effect of *H. pylori* eradication on promotor CpG island hypermethylation in gastric carcinogenesis.

In this study, we postulated that *H. pylori* infection might cause aberrant DNA hypermethylation of 3 gastric cancer-related genes (p16, CDH1, and Runt-related transcription factor 3 [RUNX-3]), which were all tumor suppressor genes.<sup>7-9</sup> Eradication of *H. pylori* might reverse methylation of these genes over the long term. We investigated methylation of the p16, CDH1, and RUNX-3 genes in gastric mucosa from patients with gastric adenoma or early gastric cancer (EGC) before and after eradication of *H. pylori* at 1-year follow-up.

## II. Materials and Methods

### 1. Patients and Study Design

In this study, gastric tissues were obtained from samples that were previously collected for another study.<sup>10</sup> *H. pylori*-positive patients with gastric adenoma or EGC who underwent endoscopic resection were enrolled. According to *H. pylori* eradication after endoscopic resection, the participants were randomly assigned to *H. pylori* eradication or non-eradication group. Patients in the eradication group received omeprazole, 20 mg; amoxicillin, 1 g; and clarithromycin, 500 mg, twice daily for 1 week. Patients in the non-eradication group received no antibiotics.<sup>10</sup> All patients underwent follow-up endoscopic examination regularly at one year intervals. Successful eradication for *H. pylori* was confirmed in the eradication group by both histologic examination and rapid urease test. *H. pylori* status was considered positive if the result of 1 or both tests (histology or rapid urease test) was positive. Negative in both histology and rapid urease test deemed *H. pylori*-negative. We used samples from that study to evaluate the effect of *H. pylori* eradication

on hypermethylation of genes before and 1 year after endoscopic resection. *H. pylori*-negative dyspepsia patients without adenoma or EGC were enrolled as normal control. All patients gave informed consent and the institutional review board of Seoul National University Hospital approved this study (H-1008-115-329).

## 2. Tissue Collection

In Biopsy samples were taken from the lesser curvature of the antrum and the lesser curvature of the body for evaluation of *H. pylori*, rapid urease test, gastric atrophy, intestinal metaplasia (IM), and DNA methylation study. The degree of atrophic gastritis (AG) and IM in the gastric mucosa was classified according to the updated Sydney system.<sup>11</sup> Negative AG/IM was considered as no evidence of AG/IM in both antrum and body. AG/IM was considered positive if the result of either antrum or body was positive. *H. pylori* density, neutrophilic inflammation activity, and mononuclear inflammation were also evaluated according to updated Sydney system.

### 3. DNA Extraction and Bisulfite Modification

Biopsy specimens obtained from non-tumorous mucosa were stored at  $-80^{\circ}\text{C}$ . DNA was extracted and verified. Specimens were homogenized in proteinase K solution (20 mmol/L Tris-hydrochloride [pH 8.0], 10 mmol/L EDTA, 0.5% sodium lauryl sulfate, and 10 mg/mL proteinase K) and then maintained for over 3 h at  $50^{\circ}\text{C}$ . DNA was separated from homogenates by phenol/chloroform extraction and ethanol precipitation.<sup>12</sup> Genomic DNA was modified by sodium bisulfite to convert unmethylated cytosines to uracil using the EZ DNA Methylation Kit (Zymo Research, Orange, CA, USA) according to the manufacturer's protocol.

## 4. MethyLight Assay

The methylation assay of the p16, CDH1, and RUNX-3 genes from bisulfite modified DNA samples was performed by using real-time PCR-based quantitative MethyLight technology.<sup>13-15</sup> Primers and probe for sequencing have been described.<sup>16</sup> In short, 2 sets of primers and probes designed to bond to bisulfite-converted DNA were used: One set of primers and a probe were used for methylated reaction, and another set was utilized as the reference locus. The DNA methylation of each examined marker was quantified and reported as a percent of methylated reference (PMR; degree of methylation). PMR was calculated as  $100 \times ((\text{methylated reaction/ALU})_{\text{sample}} / (\text{methylated reaction/ALU})_{\text{M.SssI-reference}})$ .<sup>17</sup> We considered a CpG island locus methylated if the PMR value was  $> 4$ .<sup>15</sup>

## 5. Statistical Analysis

Because the data were not normally distributed, comparison of hypermethylation rate of gene between before and at 1 year after was performed by using McNemar test. Comparison the median methylation value (PMR; percentage of methylated reference) at baseline and at one year was compared with a Wilcoxon signed-rank test. Other comparisons used the Mann-Whitney U test and Fischer's exact test. Null hypotheses of no difference were rejected if p-values were less than 0.05. Statistical calculations were done using R version 2.15.3 (R foundation for Statistical Computing, Vienna, Austria).

### III. Results

#### 1. Subject characteristics

Eighty-three patients were enrolled in the study; 31 patients in H. pylori eradication group, 34 patients in non-eradication group, or 18 patients in normal control. There were no significant differences in baseline clinicopathological variables among the groups (Table 1). Median age was 60 years old (interquartile range 50–66) and male was 67.7%. Most patients had AG/ IM while only 2 patients (3%) had no AG or IM. Half of patients had EGC and remaining half had gastric adenoma. On the other hand, most of normal control group had no AG/IM or mild AG/IM.



## 2. MethyLight Assay

In the *H. pylori* eradication group, rate of hypermethylation of p16, CDH1, and RUNX-3 genes at baseline was 80.6% (25/31), 80.6% (25/31), and 48.4% (15/31), respectively. At 1 year after eradication, methylation was 58.1% (18/31), 61.3% (19/31), and 67.7% (21/31), respectively (Table 2). There was no statistical difference in hypermethylation rates between baseline and 1 year after eradication. We also analyzed quantitative methylation value of individual gene, which was possible owing to MethyLight assay. We found that median methylation value (PMR; percentage of methylated reference) of p16 and CDH1 was significantly decreased at 1 year after eradication. For p16 gene, median baseline PMR decreased significantly from 11.7 to 5.7 ( $p=0.004$ ). For CDH1, median PMR was decreased significantly from 47.9 to 7.0 ( $p=0.001$ ). Conversely, RUNX-3 did not show any difference in methylation value (Figure 1).

In the non-eradication group, rates of methylated 3 genes were p16, 71.1%; CDH1, 97.4%; and RUNX-3, 55.3%. There were no significant changes in both hypermethylation rate and median methylation value at one-year follow-up.

Baseline p16, CDH1, and RUNX-3 genes had significantly higher methylation levels in *H. pylori* positive patients than in *H. pylori*-negative normal control (10%, 44%, 16%, respectively,  $p < 0.05$ ) (Table 2).

We observed significant decrease in both neutrophilic and mononuclear inflammation in the gastric mucosa 1 year after *H. pylori* eradication ( $p=0.01$ ). Conversely, there was no change of the inflammation in the non-eradication group (data not shown). We tried to explain why some of eradicated patients still showed CDH1 and/or p16 hypermethylation even after the eradication. We compared patients who showed decreased methylation level of CDH1/p16 after the eradication with those did not in terms of initial *H. pylori* density, initial neutrophilic/mononuclear inflammation activity, or inflammation activity 1 year after eradication. We found no significant difference of inflammation activity or *H. pylori* density between groups.

We also analyzed methylation rates and median methylation level according to gastric adenoma or EGC type. In the eradication group, significant change in median methylation level of p16 was predominant in the EGC (PMR, 15.3 [baseline], 3.2 [1 year],  $p=0.02$ ) rather than gastric adenoma (PMR, 9.7 [baseline], 5.7 [1 year];

p=0.20) (Table 3). We evaluated median methylation level of p16 in the EGC patients according to differentiation type (well, moderately, poorly differentiated adenocarcinoma, or signet ring cell carcinoma). Initial methylation level of p16 was not different between well/moderately differentiated EGC and poorly differentiated/signet ring cell EGC. However, only well or moderately differentiated EGC showed significant decrease in the methylation level of p16 after eradication (p=0.01). In terms of CDH1, significant change in PMR was found in EGC as well as adenoma. However, in the non-eradication group, there was no significant difference in methylation rate according to gastric adenoma or EGC.

## IV. Discussion

In this study, the methylation levels in three genes (p16, CDH1, and RUNX-3) were evaluated from non-neoplastic gastric mucosae using quantitative real-time PCR, MethyLight assay. Baseline p16, CDH1, and RUNX-3 genes showed significantly higher methylation levels in *H. pylori*-positive patients than in *H. pylori*-negative normal control group. Some articles reported that patients with *H. pylori* had hypermethylation of p16 and CDH1 in 46–80%, while normal participants without *H. pylori* had no/little hypermethylation,<sup>8,18</sup> which was in line with our study. However, we should consider that not all patients with *H. pylori* infection had hypermethylation of p16 and CDH1 although hypermethylation of these genes are associated with *H. pylori* infection. Previous studies showed that rates of hypermethylation were variable, ranging from 30% to 82%.<sup>8,19</sup> In addition, some studies reported methylation of MLH1 and MGMT did not decrease significantly after *H. pylori* eradication.<sup>8,20</sup> Conversely, recent study showed that MGMT methylation was significantly reduced after *H. pylori* eradication in patients with *H. pylori* gastritis (from 70 to 48%).<sup>21</sup> These contradictory results suggest that exact

mechanism still needs to be determined although researchers found epigenetic alteration associated with *H. pylori* in gastric carcinogenesis.

MethyLight is a sodium-bisulfite-dependent, quantitative, real-time PCR method to sensitively detect and quantify DNA methylation in genomic DNA. Methylation specific polymerase chain reaction (MSP) after sodium bisulfite conversion is used to determine DNA methylation status. However, due to its qualitative nature of assay, MSP cannot distinguish level of methylation.<sup>15</sup> The high sensitivity and specificity of MethyLight assay allows detection of low-frequency DNA methylation markers. The advantages of MethyLight technology include its quantitative and high-throughput nature and relatively simple assay.<sup>14</sup> Therefore, MethyLight allows better detection of DNA and less normalization errors caused by copy number changes.<sup>15</sup>

Research of the effect of *H. pylori* eradication on the CpG hypermethylation of genes in gastric mucosa typically analyzed hypermethylation of genes at six to eight weeks after *H. pylori* eradication.<sup>8,18,21,22</sup> We postulated that some genes need longer period, not 8 weeks, for reversal of CpG hypermethylation after *H. pylori*

eradication. We also assumed some genes were more likely to remain methylated once their CpG island was methylated, so eradication might have little impact. We aimed to determine whether *H. pylori* eradication affect methylation of relevant genes over the long-term.

In this study, p16 and CDH1 hypermethylation decreased in 58.1% and 61.3% of the patients, and the median values of methylation were reduced significantly at 1-year after *H. pylori* eradication compared with non-eradication group.

Hypermethylation of these genes is associated with *H. pylori* and suppressed by *H. pylori* eradication.<sup>8</sup> Specifically, CDH1 and p16 methylation were significantly decreased after *H. pylori* eradication,<sup>8,18</sup> which was inconsistent with our study. This result might stem from different patient group. In this study, patients had gastric adenoma/ EGC and most patients had AG/IM, while previous studies had no gastric adenoma or EGC.<sup>8,18</sup> However, not all patients had experienced reduction of hypermethylation of relevant genes by *H. pylori* eradication.<sup>8,18</sup> Approximately 22–76% of patients who underwent eradication had still hypermethylation of CDH1 gene and 18% of patients had still p16 hypermethylation.<sup>8,18</sup>

In this study, hypermethylation of RUNX-3 did not change one

year after *H. pylori* eradication in this study. However, some methylation profiles induced by *H. pylori* infection can be persistent even after eradication.<sup>12</sup> Several studies suggested RUNX-3 methylation as a risk factor for gastric carcinogenesis in patients with *H. pylori* infection.<sup>23,24</sup>

One study suggested that epigenetic events and gene methylation were not evenly distributed throughout the gastric mucosa, so multiple biopsies in different parts of the stomach should be performed to determine methylation status.<sup>8</sup> In this study, one biopsy was used for methylation status, which might be a limitation. Another limitation was the relatively small number of participants.

This study did not find new gastric cancer-related genes which are related to *H. pylori*, which was another limitation.

## V. Conclusion

H. pylori infection is associated with promotor methylation of genes in gastric carcinogenesis. H. pylori eradication might reverse CDH1 and p16 methylation levels. Further studies are warranted to determine long-term effect of H. pylori eradication on DNA methylation.



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Table 1. Clinicopathological characteristics among the three groups

Characteristic	Group			
	<i>Helicobacter pylori</i> eradication	Non eradication	p value <sup>a</sup>	Normal control
Gender			1.00	
Male	21 (67.7)	22 (64.7)		11 (61.1)
Female	10 (32.3)	12 (35.3)		7 (38.9)
Age (yr)	60.0 (50.0-66.0)	59.5 (54.7-66.0)	0.68	58.5 (50.0-67.5)
Gastric atrophy			0.42	
Absent	5 (16.1)	2 (5.9)		7 (38.9)
Mild	6 (19.4)	6 (17.6)		9 (50.0)
Moderate	11 (35.5)	10 (29.4)		2 (11.1)
Marked	3 (9.7)	6 (17.6)		0 (0)
NA	6 (19.4)	10 (29.4)		0 (0)
Intestinal metaplasia			1.00	
Absent	1 (3.2)	2 (5.9)		11 (61.1)
Mild	9 (29.0)	8 (23.5)		3 (16.7)
Moderate	13 (41.9)	19 (55.9)		4 (22.2)
Marked	8 (25.8)	5 (14.7)		0 (0)
Gastric atrophy or IM			0.97	
Absent	1 (3.2)	1 (2.9)		7 (38.9)
Mild	9 (29.0)	9 (26.5)		9 (50.0)
Moderate	12 (38.7)	15 (44.1)		2 (11.1)
Marked	9 (29.0)	9 (26.5)		0 (0)
<i>H. pylori</i> density			0.75	0 (0)
Mild	10 (32.3)	14 (41.2)		
Moderate	14 (45.2)	13 (38.2)		
Marked	7 (22.6)	7 (20.6)		
Neutrophilic inflammation			0.25	
Absent	1 (3.2)	0 (0)		
Mild	0 (0)	3 (8.8)		
Moderate	25 (80.6)	27 (79.4)		
Marked	5 (16.1)	4 (11.8)		
Mononuclear inflammation			0.43	
Absent	0 (0)	0 (0)		
Mild	0 (0)	0 (0)		
Moderate	20 (64.5)	25 (73.5)		
Marked	11 (35.5)	9 (26.5)		
Histology			0.55	
Low-grade dysplasia	12 (38.7)	10 (29.4)		0 (0)
High-grade dysplasia	4 (12.9)	3 (8.8)		0 (0)
Adenocarcinoma	15 (48.4)	21 (61.8)		0 (0)
Well differentiated	5 (16.1)	11 (32.4)		
Moderately differentiated	7 (22.6)	6 (17.6)		
Poorly differentiated	2 (6.5)	1 (2.9)		
Signet ring cell	1 (3.2)	3 (8.8)		
Total	31 (100)	34 (100)		18 (100)

<sup>a</sup>Comparison between *H. pylori* eradication and non-eradication group

IM, intestinal metaplasia; NA, not available.

Table 2. Promotor gene methylation rates and median methylation values at baseline and one year among groups

Variable	Group							
	<i>Helicobacter pylori</i> eradication			Non-eradication			Normal control	
	Baseline	1 yr	p-value <sup>a</sup>	Baseline	1 yr	p-value <sup>b</sup>	Baseline	p-value <sup>c</sup>
Methylated gene (%)								
P16	80.6	58.1	1.00	71.1	84.2	0.33	10.0	0.01
RUNX-3	48.4	67.7	0.25	55.3	65.8	0.05	16.0	0.03
CDH1	80.6	61.3	1.00	97.4	100	1.00	44.0	0.01
Median methylation value (PMR)								
P16	11.7	5.7	0.01	12.9	17.8	0.25	3.4	0.01
RUNX-3	2.2	9.8	0.41	30.5	50.4	0.18	13.9	0.01
CDH1	47.9	7.0	0.01	67.7	76.6	0.24	5.3	0.01
Patient (total, n)	31			34			18	

PMR, percentage of methylated reference.

<sup>a</sup>Comparison of methylation between baseline and one year after in the *H. pylori* eradication group.

<sup>b</sup>Comparison of methylation between baseline and one year after in the non-eradication group.

<sup>c</sup>Comparison of baseline methylation in the eradication group with normal control.

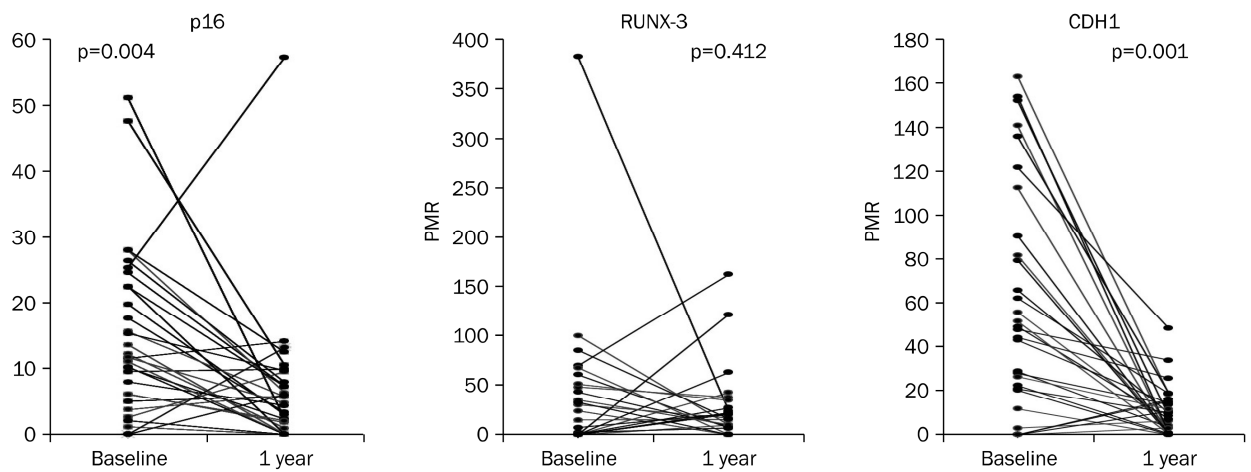
Table 3. Promotor gene methylation rates and median methylation values at baseline and one year according to gastric adenoma or early gastric cancer

Variable	<i>Helicobacter pylori</i> eradication						Non-eradication					
	Adenoma			Cancer			Adenoma			Cancer		
	Baseline	1 yr	p-value	Baseline	1 yr	p-value	Baseline	1 yr	p-value	Baseline	1 yr	p-value
Methylated gene (%)												
P16	75.0	68.8	1.00	86.7	46.7	0.07	61.5	69.2	1.00	76.2	95.2	0.13
RUNX-3	62.5	75.0	0.73	33.3	60.0	0.13	61.5	69.2	1.00	52.4	71.4	0.29
CDH1	81.3	56.3	0.29	80.0	66.7	0.69	100	100	1.00	95.2	100	1.00
Median methylation value (PMR)												
P16	9.7	5.7	0.20	15.3	3.2	0.02	5.24	10.4	0.55	6.92	14.4	0.06
RUNX-3	19.1	15.9	0.61	0.01	9.6	0.96	11.3	22.2	0.02	9.1	25.4	0.59
CDH1	49.7	2.4	0.01	28.9	9.7	0.04	44.0	99.7	0.04	78.1	78.3	0.59
Patient (n)	16			15			13			21		

PMR, percentage of methylated reference



Figure 1. Changes in quantitative value of MethyLight assay (PMR; percentage of methylated reference) of p16, RUNX-3 and CDH1 at baseline and after one year in the *H. pylori* eradication group. PMR value in p16 and CDH1 were significantly reduced one year after eradication





## 국문초록

### 헬리코박터 제균 치료와 위암 관련 유전자의 CpG island 과염기화 억제

서론: 헬리코박터 파일로리 감염은 위점막의 DNA 과염기화를 유발시킨다. 헬리코박터 제균치료가 위암 발생에 중요한 유전자의 DNA 과염기화를 억제하는지에 대해 알아보하고자 하였다.

방법: 헬리코박터 파일로리 균 양성이면서 위 선종 또는 위암으로 내시경 절제술을 시행한 환자를 대상군으로 하였다. 헬리코박터 제균 치료군과 비치료군으로 무작위 배정하여 제균 치료군은 아목시실린, 프로톤 펌프 억제제, 클레리스로마이신 하루 2회 7일간 처방 받았고 비치료군은 치료제를 처방 받지 않았다. 헬리코박터 파일로리 균 음성이면서 정상인 사람을 정상 대조군으로 하였다. CpG 과염기화 유전자는 p16, CDH1, RUNX-3이며 위암 발생과 연관이 있고 종양 억제 유전자이다. MethyLight 분석을 통해 과염기화를 정량적으로 검사하였다. 헬리코박터 제균 치료 전과 치료 후 1년 후의 유전자의 DNA 과

염기화 정도를 비교하였다.

결과: 헬리코박터 파일로리 양성 환자군에서 유전자의 DNA 과염기화는 p16은 80.6%, CDH1은 80.6%, RUNX-3는 48.4%에서 관찰되었다. 정상 대조군에서는 p16은 10%, CDH1은 44%, RUNX-3는 16%에서만 과염기화가 관찰되었고 양성 환자군에 비해 유의한 차이를 보였다. 헬리코박터 제균치료군에서 1년 후의 p16과 CDH1은 58.1%, 61.3%로 감소를 보였으나 RUNX-3는 차이를 보이지 않았다.

결론: 헬리코박터 파일로리 감염은 위암 발생에 중요한 유전자의 과염기화와 연관되어 있다. 헬리코박터 제균치료는 p16과 CDH1의 과염기화를 억제시킨다.

주요어: 헬리코박터 파일로리, 제균치료, CpG 과염기화, p16, CDH1, 위암발생

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